

CCN1 COMPOSITIONS AND METHODS

FIELD OF THE INVENTION

[0001] The present invention relates to materials and methods involving extracellular matrix signaling molecules in the form of polypeptides involved in cellular responses to growth factors. More particularly, the invention is directed to CCN1-related peptides, compositions thereof, and methods of using these polypeptides. The invention is also directed to anti-CCN1 antibodies.

BACKGROUND

[0002] Angiogenesis, or the formation of new blood vessels from pre-existing ones, is a complex process requiring the coordinated execution of multiple cellular events. *See* Risau (1997) *Nature* 386, 671-674. The sprouting of vessels requires degradation of the basement membrane surrounding the parental vessel, migration of vascular endothelial cells towards the angiogenic stimulus, proliferation of endothelial cells and their alignment into tubular structures, and coalescence of new vessels into circular loops to provide blood supply to the target tissue. *See* Risau. Angiogenesis is essential for embryogenesis, and in the adult, it is important in the female reproductive cycle and in wound healing. Angiogenesis may underlie a number of pathological conditions including diabetic retinopathy, arthritis, arteriosclerosis, psoriasis, and cancer. *See* Folkman (1995) *Nature Medicine* 1, 27-31. It is now clear that angiogenesis is regulated by a network of multiple inducers and inhibitors. *See* Bouck *et al.* (1996) *Adv. Cancer Res.* 69, 135-174 and Davis *et al.* (1999) *Curr. Top. Microbiol. Immunol.* 237, 173-185.

[0003] The CCN family of matricellular proteins are cysteine-rich, secreted proteins that are associated with the extracellular matrix (ECM) but serve regulatory rather than structural functions. Members of the CCN family, which include CCN1 (CYR61), CCN2 (CTGF), CCN3 (NOV), CCN4 (WISP-1), CCN5 (WISP-2), and CCN6 (WISP-3) (*See* Brigstock (1999) *Endocr. Rev.* 20, 189-206; Lau *et al.* (1999) *Exp. Cell Res.* 248, 44-57), are composed of an N-terminal secretory signal peptide followed by four conserved domains with homology to insulin-like growth factor binding protein, von Willebrand factor type C repeat, thrombospondin type 1 repeat, and a C-terminal domain (CT) with heparin-binding motifs and sequence similarity to the C-termini of von Willebrand factor and mucin. *See* Bork (1993) *FEBS Lett.* 327, 125-130. In

keeping with their homology to ECM proteins and localization to the ECM, several CCN proteins have been shown to support cell adhesion, induce focal adhesion complexes and stimulate adhesive signaling. See Kireeva *et al.* (1996) *Mol. Cell. Biol.* **16**, 1326-1334; Chen *et al.* (2001) *J. Biol. Chem.* **276**, 10443-10452; Chen (2001) *J. Biol. Chem.* **276**, 47329-47337.

[0004] Among members of the CCN family, CCN1 and CCN2 have been most extensively characterized. Both proteins stimulate cell migration, promote cell survival, and augment growth factor-induced mitogenesis. See Kireeva *et al.* (1997) *Exp. Cell Res.* **233**, 63-77; Jedsadayanmata *et al.* (1999) *J Biol.Chem.* **274**, 24321-24327; Babic *et al.* (1999) *Mol. Cell. Biol.* **19**, 2958-2966; Schober *et al.* (2002) *Blood* **99**, 4457-4465; Leu *et al.* (2002) *J.Biol.Chem.* **277**, 46248-46255. Both proteins are known to induce angiogenesis and chondrogenesis; See Babic *et al.*; Wong *et al.* (1997) *Dev.Biol.* **192**, 492-508; Babic *et al.* (1998) *Proc. atl. Acad. Sci. U.S.A.* **95**, 6355-6360; Shimo *et al.* (1999) *J.Biochem.(Tokyo.)* **126**, 137-145; Ivkovic *et al.* (2003) *Development* **130**, 2779-2791. Although CCN proteins do not contain a RGD sequence motif, both CCN1 and CCN2 are direct ligands of multiple integrin receptors, which mediate many of their activities. See Jedsadayanmata *et al.* (1999) *J Biol.Chem.* **274**, 24321-24327; Schober *et al.* (2002) *Blood* **99**, 4457-4465; Leu *et al.* (2002) *J.Biol.Chem.* **277**, 46248-46255; Kireeva *et al.* (1998) *J.Biol.Chem.* **273**, 3090-3096; Chen *et al.* (2000) *J.Biol.Chem.* **275**, 24953-24961; Grzeszkiewicz *et al.* (2001) *J Biol.Chem.* **276**, 21943-21950; Grzeszkiewicz *et al.* (2002) *Endocrinology* **143**, 1441-1450. Targeted disruption of the *CCN1* gene in mice resulted in embryonic lethality due to vascular defects, whereas *CCN2*-null mice die perinatally due to respiratory failure as a consequence of skeletal malformation. See (Mo *et al.* (2002) *Mol. Cell Biol.* **22**, 8709-8720); Ivkovic *et al.* (2003) *Development* **130**, 2779-2791. These findings indicate that members of the CCN family serve essential and non-redundant functions during development.

[0005] CCN1 (cysteine-rich 61, CYR61), an angiogenic inducer encoded by a growth factor-inducible immediate-early gene, is a novel integrin ligand whose expression is essential for proper embryonic development. Recent studies by targeted disruption of the *CCN1* gene in mice shows that *CCN1*-null embryos suffer embryonic death due primarily to vascular defects in both the placenta and the embryo. See Mo *et al.* (2002) *Mol. Cell Biol.* **22**, 8709-8720. In addition to embryonic angiogenesis, CCN1 may also promote pathological angiogenesis under such conditions as tumor growth and wound healing. Stable transfection of *CCN1* in tumor cell lines

that do not otherwise express *CCN1* enhances tumorigenicity with an increased vascularization of the *CCN1*-expressing tumors. See Babic *et al.* (1998) *Proc. Natl. Acad. Sci. U.S.A.* **95**, 6355-6360; Xie *et al.* (2001) *J. Biol. Chem.* **276**, 14187-14194; Tsai *et al.* (2002) *Oncogene* **21**, 8178-8185. Furthermore, estrogen-induced *CCN1* expression has been associated with advanced human breast cancer. See Xie *et al.* (2001) *Cancer Res.* **61**, 8917-8923; Sampath *et al.* (2001) *Endocrinology* **142**, 2540-2548; Tsai *et al.* (2000) *Cancer Res.* **60**, 5603-5607. Overexpression of *CCN1* has also been observed in restenosed blood vessels and advanced atherosclerotic lesions, underscoring its pathologic importance in vascular diseases. See Hilfiker *et al.* (2002) *Circulation* **106**, 254-260; Wu *et al.* (2000) *Int. J. Mol. Med.* **6**, 433-440; Grzeszkiewicz *et al.* (2002) *Endocrinology* **143**, 1441-1450; Schober *et al.* (2002) *Blood* **99**, 4457-4465. In addition, the expression of *CCN1* in cutaneous healing wounds, coupled with its ability to activate a genetic program for wound healing in human skin fibroblasts, suggests an important role for *CCN1* in injury repair. See Latinkic *et al.* (1991) *Nucleic Acids. Res.* **19**, 3261-3267; Chen *et al.* (2001) *J. Biol. Chem.* **276**, 47329-47337.

[0006] Upon synthesis, *CCN1* is secreted and becomes associated with the cell surface or the ECM. See Yang *et al.* (1991) *Cell Growth & Differentiation* **2**, 351-357. Previous studies have shown that *CCN1* supports cell adhesion, induces cell migration, enhances growth factor-induced mitogenesis, and promotes cell survival under apoptotic conditions. See Kireeva *et al.* (1996) *Mol. Cell. Biol.* **16**, 1326-1334; Leu *et al.* (2002) *J. Biol. Chem.* **277**, 46248-46255. These cellular activities of *CCN1* can be attributed in part to its ability to interact with integrin adhesion receptors. To date, five integrins, $\alpha_6\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_{IIb}\beta_3$, and $\alpha_M\beta_2$, have been identified as *CCN1* receptors in various cell types. See Schober *et al.* (2002) *Blood* **99**, 4457-4465; Kireeva *et al.* (1998) *J. Biol. Chem.* **273**, 3090-3096; Jedsadayanmata *et al.* (1999) *J. Biol. Chem.* **274**, 24321-24327; Grzeszkiewicz *et al.* (2001) *J. Biol. Chem.* **276**, 21943-21950; Chen *et al.* (2000) *J. Biol. Chem.* **275**, 24953-24961. In an earlier study, we have demonstrated that *CCN1* induces neovascularization in the rat corneal micropocket assay. See Babic *et al.* (1998) *Proc. Natl. Acad. Sci. U.S.A.* **95**, 6355-6360. Consistent with these *in vivo* findings, *CCN1* promotes tubule formation of human umbilical vein endothelial cells (HUVECs) in a collagen gel assay, and this process is dependent on integrins $\alpha_6\beta_1$ and $\alpha_v\beta_3$. See Leu *et al.* (2002) *J. Biol. Chem.* **277**, 46248-46255.

[0007] Integrin $\alpha_6\beta_1$ has been shown to mediate a number of CCN1 activities in several cell types. CCN1 supports fibroblast adhesion through interaction with integrin $\alpha_6\beta_1$ and cell surface heparin sulfate proteoglycans (HSPGs), leading to extensive formation of filopodia and lamellipodia with $\alpha_6\beta_1$ -containing focal complexes localized at leading edges of the pseudopods. See Chen *et al.* (2001) *J. Biol. Chem.* **276**, 10443-10452. Moreover, integrin-dependent outside-in signaling are induced resulting in the activation of focal adhesion kinase, paxillin, Rac, and mitogen-activated protein kinases, and upregulation of several angiogenic regulators. See Chen *et al.* (2001) *J. Biol. Chem.* **276**, 47329-47337; Chen *et al.* (2001) *J. Biol. Chem.* **276**, 10443-10452. In addition to fibroblasts, CCN1 also interacts with integrin $\alpha_6\beta_1$ on vascular smooth muscle cells and vascular endothelial cells. See Grzeszkiewicz *et al.* (2002) *Endocrinology* **143**, 1441-1450; Leu *et al.* (2002) *J. Biol. Chem.* **277**, 46248-46255. Recently, we have shown that pro-angiogenic activities of CCN1 are differentially mediated through integrin $\alpha_6\beta_1$ and $\alpha_v\beta_3$ in unactivated and activated HUVECs, respectively. Leu *et al.* (2002) *J. Biol. Chem.* **277**, 46248-46255.

[0008] In addition to CCN1, other members of the CCN family include CCN2 (connective tissue growth factor, CTGF), CCN3 (nephroblastoma-overexpressed, NOV), and the Wnt-inducible secreted proteins CCN4 (WISP-1), CCN5 (WISP-2), and CCN6 (WISP-3)(22-24). CCN proteins are organized into four distinct modular domains: I) an insulin-like growth factor binding protein homology domain, II) a von Willebrand factor (vWF) type C repeat domain, III) a thrombospondin type I repeat (TSP1) domain, and IV) a carboxyl terminal (CT) domain with heparin binding motifs and sequence similarities to the C-termini of vWF and mucins (see Fig. 1A). Several CCN proteins have been shown to interact with multiple integrins, and therefore, localization of the integrin binding sites in CCN proteins will provide new insights into the structure-function relationship of this newly established family of matricellular proteins. We previously found that a truncated CCN1 lacking the C-terminal domain is capable of inducing smooth muscle cell migration through integrin $\alpha_6\beta_1$. See Grzeszkiewicz *et al.* (2001) *J Biol. Chem.* **276**, 21943-21950. These findings suggest that the integrin $\alpha_6\beta_1$ binding site(s) resides within the first three domains of CCN1. We herein identify a novel 17-residue sequence, designated T1, in the CCN1 thrombospondin type I repeat domain that mediates $\alpha_6\beta_1$ -dependent cell adhesion. By affinity chromatography, we demonstrate direct interaction of $\alpha_6\beta_1$ with the T1

sequence. In addition, we demonstrate that through a coreceptor complex of $\alpha_6\beta_1$ and heparin sulfate proteoglycans (HSPGs), activity of CCN1 is affected by heparin binding sites H1 and H2 as well as T1. The heparin binding sites H1 and H2 are required for prolonged activation of MAPKs as well as upregulation of *Vegf* and MMP-1 expression, which are relevant to angiogenesis and matrix metabolism. We also demonstrate that synthetic peptides derived from the T1 sequence specifically block $\alpha_6\beta_1$ -dependent cell adhesion, our newly identified $\alpha_6\beta_1$ binding site in CCN1 may serve as a basis for the development of antagonists to integrin $\alpha_6\beta_1$. This newly identified $\alpha_6\beta_1$ binding site in CCN1 may serve as a basis for the development of antagonists to integrin $\alpha_6\beta_1$.

SUMMARY

[0009] The present invention provides extracellular matrix (ECM) signaling molecule-related materials and methods. In particular, the present invention is directed to CCN1-related peptides, compositions thereof, and methods of using these polypeptides. The invention is also directed to anti-CCN1 antibodies.

[0010] One aspect of the present invention relates to a CCN1 fragment comprising a sequence selected from the group consisting of amino acids 224-240 of murine CCN1, amino acids 231-240 of murine CCN1, amino acids 226-242 of human CCN1, and amino acids 233-242 of human CCN1. The CCN1 fragment may comprise from 8 to 50 amino acids. The present invention also relates to variants, analogs, homologs or derivatives of the CCN1 fragments.

[0011] Another aspect of the present invention relates to a method of screening for a modulator of angiogenesis comprising contacting a test biological sample capable of undergoing angiogenesis with an ECM signaling molecule and a suspected modulator. As a control, a second biological sample is also contacted with an ECM signaling molecule. A modulator of angiogenesis is identified by the ability to alter the level of angiogenesis in the test sample. The ECM signaling molecule may be a CCN1 fragment or a fragment, variant, analog, homolog or a derivative thereof. Another aspect of the present invention relates to a modulator identified by the present method.

[0012] Another aspect of the present invention relates to a method of screening for a modulator of angiogenesis comprising implanting a test implant into a test animal, wherein the test implant

comprises a suspected modulator and an ECM signaling molecule. As a control, a second implant comprising an ECM signaling molecule is implanted into a test animal, which may be the same animal or a different test animal. A modulator of angiogenesis is identified by its ability to alter the level of blood vessel development in the test implant when compared to the control sample. The ECM signaling molecule may be a CCN1 fragment or a fragment, variant, analog, homolog or a derivative thereof. Another aspect of the present invention relates to a modulator identified by the present method.

[0013] Another aspect of the present invention relates to a method of screening for a modulator of oncogenesis comprising contacting a tumor with a suspected modulator along with an ECM signaling molecule. As a control, a second tumor is also contacted with an ECM signaling molecule. A modulator of oncogenesis may be identified by its ability to alter the level of oncogenesis of the test tumor when compared to the control tumor. The ECM signaling molecule may be a CCN1 fragment or a fragment, variant, analog, homolog or a derivative thereof. Another aspect of the present invention relates to a modulator identified by the present method.

[0014] Another aspect of the present invention relates to a method of screening for a modulator of cell adhesion comprising contacting a test biological sample on a surface compatible with cell adherence with a suspected modulator along with an ECM signaling molecule. As a control, a second biological sample on a surface compatible with cell adherence is also contacted with an ECM signaling molecule. A modulator of cell adhesion is identified by its ability to alter the level of cell adhesion of the test sample when compared to the control sample. The ECM signaling molecule may be a CCN1 fragment or a fragment, variant, analog, homolog or a derivative thereof. Another aspect of the present invention relates to a modulator identified by the present method.

[0015] Another aspect of the present invention relates to a method of screening for a modulator of cell migration comprising seeding cells capable of undergoing cell migration onto a test gel matrix comprising a suspected modulator and an ECM signaling molecule. As a control, cells capable of undergoing cell migration are also seeded onto a second biological sample gel matrix comprising an ECM signaling molecule. A modulator of cell adhesion may be identified by its ability to alter the level of cell migration in the test matrix when compared to the control matrix.

The ECM signaling molecule may be a CCN1 fragment or a fragment, variant, analog, homolog or a derivative thereof. Another aspect of the present invention relates to a modulator identified by the present method.

[0016] Another aspect of the present invention relates to an antibody that specifically binds to a CCN1 fragment, or a variant, analog, homolog or derivative of said CCN1 fragment. Another aspect of the present invention relates to a composition comprising an antibody that specifically binds to a CCN1 fragment, or a variant, analog, homolog or derivative of said CCN1 fragment.

BRIEF DESCRIPTION OF THE DRAWINGS

[0017] Fig. 1 demonstrates the protein purity of recombinant CCN1 domain fragments and their ability to support cell adhesion. Recombinant CCN1 domain fragments were produced as hexahistidine-tagged fusion proteins by a baculovirus-expression system and purified by chromatography on cobalt-agarose. *A*, a schematic representation of the structural domains of full-length CCN1 and the isolated domain fragments. The T1 sequence in domain III (TSP1 domain) is indicated by the shaded area. *B*, recombinant CCN1 domain fragments and full-length CCN1 (2 μ g) were electrophoresed on 15% SDS-polyacrylamide gel and detected by Coomassie Blue staining. *C*, the resolved proteins were subjected to immunoblotting with polyclonal anti-CCN1 antibodies. See Kireeva *et al.* (1996) *Mol. Cell. Biol.* 16, 1326-1334. Molecular mass markers are indicated in kDa on the left. *D*, maleic anhydride Reacti-Bind microtiter wells were coated with purified recombinant CCN1 domain fragments or bovine serum albumin (BSA) (50 μ g/ml, 50 μ l/well) overnight at 4°C and blocked with 1% BSA. Protein coating efficiency was detected by an enzyme-linked immunosorbent assay (ELISA) using an anti-polyhistidine monoclonal antibody (mAb). *E*, washed 1064SK fibroblasts, resuspended in serum-free medium, were plated onto wells (3 x 10⁴ cells per well) precoated with CCN1 (20 μ g/ml) or the indicated domain fragments (50 μ g/ml). Cells were allowed to adhere for 20 min at 37°C. Adherent cells were fixed, stained with methylene blue, and quantified by absorbance at 620 nm. Data are means \pm S.D. of triplicate determinations. Panels *D* and *E* are representative of three experiments.

[0018] Fig. 2 demonstrates that Domain III (TSP1 domain) of CCN1 supports fibroblast adhesion through integrin $\alpha_6\beta_1$. Fibroblast adhesion to microtiter wells coated with full-length

CCN1 (20 $\mu\text{g/ml}$) or domain III fragment (50 $\mu\text{g/ml}$) was performed as described in the legend of Fig. 1. *A*, where indicated, cells were suspended in serum-free medium containing EDTA (2.5 mM), Mg^{++} (5 mM), Ca^{++} (5 mM), or Mn^{++} (0.5 mM) before plating. *B*, cells were preincubated with vehicle buffer (No Add), normal mouse IgG (100 $\mu\text{g/ml}$), anti- $\alpha_v\beta_3$ mAb LM609 (50 $\mu\text{g/ml}$) (Chemicon-Temecula, CA), anti- α_6 mAb GoH3 (50 $\mu\text{g/ml}$) (Immunotech-Marseille, France), or anti- β_1 mAb P4C10 (1:50 ascites) (Life Technologies/Gibco-BRL) for 60 min prior to plating. Data are means \pm S.D. of triplicate determinations and are representative of three experiments.

[0019] Fig. 3 demonstrates that recombinant GST-T1 fusion protein supports $\alpha_6\beta_1$ -dependent fibroblast adhesion. *A*, Microtiter wells were coated with 200 $\mu\text{g/ml}$ recombinant GST-peptide fusion proteins with their sequences shown in Table I. Protein coating was performed overnight at 4°C followed by blocking with 1% BSA. Fibroblast adhesion was assessed as described in the legend of Fig. 1. *B*, Cells were suspended in serum-free medium containing EDTA (2.5 mM), Mg^{++} (5 mM), Ca^{++} (5 mM), or Mn^{++} (0.5 mM) and plated onto the microtiter wells coated with glutathione S-transferase (GST) (50 $\mu\text{g/ml}$), GST-T1 (50 $\mu\text{g/ml}$), or CCN1 (1 $\mu\text{g/ml}$). *C*, cells were preincubated with vehicle buffer (No Add), normal mouse IgG (100 $\mu\text{g/ml}$), anti- $\alpha_v\beta_3$ mAb LM609 (50 $\mu\text{g/ml}$), anti- α_6 mAb GoH3 (50 $\mu\text{g/ml}$), or anti- β_1 mAb clone P4C10 (1:50 ascites) for 60 min prior to plating. Data are means \pm S.D. of triplicate determinations and are representative of three experiments.

[0020] Fig. 4 demonstrates that synthetic T1 peptide supports $\alpha_6\beta_1$ -dependent cell adhesion. *A*, microtiter wells were coated with synthetic T1, T2, T3, or T4 peptides (0.2 mM) overnight at 4°C and blocked with 1% BSA. Fibroblast were allowed to adhere to the peptide-coated wells for 20 min at 37°C. *B*, cells were preincubated with vehicle buffer (No Add) or the indicated monoclonal antibodies for 60 min prior to plating onto T1-coated wells. Data are means \pm S.D. of triplicate determinations and are representative of three experiments.

[0021] Fig. 5 demonstrates that soluble T1 peptide inhibits $\alpha_6\beta_1$ -dependent cell adhesion. *A*, microtiter wells were coated with CCN1 (1 $\mu\text{g/ml}$), CCN2 (2 $\mu\text{g/ml}$), or CCN3 (5 $\mu\text{g/ml}$) and blocked with 1% BSA. Washed fibroblasts were pre-treated with vehicle buffer (No Add) or with soluble T1, T2, T3 or T4 peptides (0.2 mM) for 30 min and plated onto wells coated with the indicated CCN proteins. *B* and *C*, various concentrations of T1 peptide were added to the cell suspension prior to plating onto wells coated with fibronectin (FN, 2 $\mu\text{g/ml}$), vitronectin

(VN, 0.4 $\mu\text{g/ml}$), type I collagen (0.5 $\mu\text{g/ml}$), laminin (LN, 5 $\mu\text{g/ml}$) or CCN1 (1 $\mu\text{g/ml}$).

Recombinant murine CCN1 protein was purified from serum-free insect cell conditioned media using the baculovirus expression system as described. *See Kireeva et al. (1996) Mol. Cell. Biol.* 16, 1326-1334. Rat Type I collagen, vitronectin, laminin, and fibronectin were purchased from Collaborative Biomedical (Bedford, MA). Cell adhesion was assayed as described in the legend of Fig. 1. Data are means \pm S.D. of triplicate determinations and are representative of three experiments.

[0022] Fig. 6 demonstrates that the TTSWSQCSKS sequence in T1 contains critical determinants for $\alpha_6\beta_1$ -dependent cell adhesion. Site-directed alanine substitutions of the T1 sequence in the T1-GST fusion protein were performed as described in Materials and Methods. Wild type T1 fusion protein (GST-T1-WT), its scrambled variant (GST-T1-Scram) or the alanine substituted mutants was coated onto microtiter wells at a protein concentration of 200 $\mu\text{g/ml}$. After blocking with 1% BSA, fibroblast adhesion proceeded as described. Results are means \pm S.D. of triplicate determinations and are representative of three experiments.

[0023] Fig. 7 demonstrates affinity purification of integrin $\alpha_6\beta_1$ from fibroblast lysates on GST-T1-coupled Affi-gel. Cell surface proteins on fibroblasts were radio-iodinated by the lactoperoxidase-glucose oxidase method as described in Materials and Methods. Labeled cells were solubilized in starting buffer containing 200 mM octylglucoside and 0.5 mM Mn^{++} . The cell lysates (lane 1) were applied to affinity columns of Affi-gel agarose coupled with GST-scrambled T1 in A or GST-T1 in B. After washing with the starting buffer (lanes 2-4), the columns were eluted with 0.35 M NaCl (lane 5-8). Proteins in the eluted fractions were resolved on 7% SDS-polyacrylamide gels under non-reducing conditions and detected by autoradiography. In C, the high salt eluates from the GST-T1 column were pooled and subjected to immunoprecipitation with anti- α_6 (GoH3), or anti- α_v (P3G8) mAb. The immunoprecipitated proteins were analyzed under non-reducing conditions. Molecular mass markers are indicated in kDa on the left. Results are representative of two experiments.

[0024] Fig. 8 demonstrates that the T1 peptide blocks CCN1-induced endothelial tubule formation in a collagen gel matrix. Unstimulated HUVECs were plated on 24-well plates precoated with type I collagen gels (2 mg/ml) in the absence (No Add) or presence of 50 $\mu\text{g/ml}$ CCN1, and a second layer of gels was overlaid on the attached cells as described in Materials

and Methods. Where indicated, cell suspension was incubated with the tested peptides for 30 min prior to plating. Tubule formation was assessed 16-20 h thereafter. Results are representative of three separate experiments (magnification x 100).

[0025] Fig. 9 depicts the construction and expression of CCN1 and mutants. A, schematic diagram of constructs of wild type CCN1 (WT) and mutants either bearing the K239E point mutation in T1 (SM), disruptions in H1 and H2 (DM), or combined mutations in T1, H1, and H2 (TM). Each construct is similarly endowed with an N-terminal secretory signal and a C-terminal FLAG epitope tag. Recombinant proteins were expressed in insect cells via a baculovirus vector. Wild type T1, H1, and H2 sequences and specific a.a. changes in the mutants are shown. B, fibroblasts were plated on microtiter wells coated with either GST, GST-T1 peptide fusion, or GST-T1(K239E) peptide fusion protein (50 μ g/ml each). Cells were allowed to adhere at 37°C for 20 min. After washing, adherent cells were fixed, stained with methylene blue, and quantified by absorbance at 620 nm. C, Coomassie brilliant blue stained 10% SDS-PAGE in which FLAG affinity-purified recombinant proteins (2 μ g each) were electrophoretically separated. Molecular mass (kDa) of markers are shown at left. The gel was immunoblotted with polyclonal anti-CCN1 antibodies and shown in the lower panel.

[0026] Fig. 10 demonstrates that fibroblast adhesion to mutant SM is heparin-sensitive. A, 1064SK human fibroblasts were plated on microtiter wells coated with the indicated amounts of recombinant WT CCN1 or SM mutant, and cell adhesion was assessed as described in Fig. 1. B, fibroblasts were untreated, or treated with either heparinase I (2 units/ml) or chondroitinase ABC (2 units/ml) prior to adhesion to microtiter wells coated with WT CCN1 (2 μ g/ml), SM (2 μ g/ml) or VN (0.5 μ g/ml). Where indicated, soluble heparin was present at 1 μ g/ml in the culture medium. Data shown are mean \pm S.D. of three determinations and representative of three experiments.

[0027] Fig. 11 demonstrates DM supported $\alpha_6\beta_1$ -mediated cell adhesion. A, fibroblasts were plated on microtiter wells coated with the indicated amounts of DM or TM and cell adhesion was evaluated. B, cells were preincubated with 40 μ g/ml of function-blocking mAb against integrin $\alpha_v\beta_3$ (LM609), integrin α_6 (GoH3), or integrin β_1 subunit (P4C10, 1:50 ascites) at room temperature for 1 h prior to plating. Cell adhesion was assessed as above. Data shown are mean \pm S.D. of three determinations and representative of three experiments.

[0028] Fig. 12 demonstrates effects of CCN1 mutants on MAPK activation and gene expression. A. MAPK activation. 1064SK fibroblasts were serum-starved and resuspended in serum-free medium at 6×10^5 cells/ml. Cells were plated on 1 ml/dish (35-mm dishes) coated with WT CCN1 (10 μ g/ml), SM (10 μ g/ml), DM (250 μ g/ml), or laminin (LN, 10 μ g/ml) for 1-5 hrs as indicated. Clarified lysates were separated on 10% SDS-PAGE, and immunoblotted with polyclonal anti-MAPK antibodies, or antibodies against dually phosphorylated active p42/p44 MAPKs. B, regulation of gene expression. Primary human skin fibroblasts were serum-starved 24 hours before being treated with 10 μ g/ml each of WT CCN1 (WT), mutant proteins (SM, DM and TM), or BSA control (B) for 24 hours and total cellular RNA was isolated. Expression of *Vegf* and MMP-1 was evaluated by RNA blotting (20 μ g of total RNA in each lane) following electrophoresis, and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression was monitored as a control.

[0029] Fig. 13 demonstrates HUVEC adhesion and migration to CCN1 mutants through integrin $\alpha_v\beta_3$. A, HUVECs detached in 2.5 mM EDTA and resuspended in serum-free medium were adhered to wells pre-coated with 15 μ g/ml of CCN1 wild type CCN1 (WT) or SM, or 50 μ g/ml of DM or TM. Where indicated, cells were treated with EDTA (5 mM), GRGDS β peptide (RGDS, 0.2 mM), anti- $\alpha_v\beta_3$ mAb LM609 (40 μ g/ml), anti- α_6 mAb GoH3 (40 μ g/ml) for 60 min prior to plating. Cell adhesion was measured as described. B, migration of HUVECs to CCN1 or mutants were evaluated using Transwell chambers. 15 μ g/ml of CCN1 WT or mutant SM, 50 μ g/ml of mutant DM or TM was immobilized on the lower surface of the Transwell membrane that separated the two chambers. HUVECs were treated with 100 nM PMA (Sigma, St. Louis, MO) for 30 min to activate integrin receptors. Where indicated, prior to plating in the upper chamber, cells were preincubated with vehicle buffer (No Add), normal mouse IgG (100 μ g/ml), GoH3 (50 μ g/ml), LM609 (50 μ g/ml) for another 30 min. Cells were allowed to migrate for 8 h, and those migrated to the lower chamber were counted in 10 random high power fields. Data shown are mean \pm S.D. of three determinations and representative of three experiments.

[0030] Fig. 14 demonstrates that CCN1 mutants may enhance VEGF-induced DNA synthesis through integrin $\alpha_v\beta_3$. HUVECs were preincubated with vehicle buffer (No Add), LM609 (25 μ g/ml), GoH3 (25 μ g/ml), or normal mouse IgG (25 μ g/ml) for 1h. Cells were then treated with VEGF (5 ng/ml) and/or CCN1 or mutant proteins (5 μ g/ml each) in the presence of

[³H]thymidine, incorporation of which was assessed 48 h thereafter. Data shown are mean \pm S.D. of three determinations and representative of three experiments.

[0031] Fig. 15 demonstrates that CCN1 mutants may promote HUVEC survival. Serum-starved HUVECs were allowed to attach to coverslips pre-coated with 20 μ g/ml laminin (LN) for 4 hr, followed by addition of serum, CCN1 or mutant proteins (5 μ g/ml each) for an additional 16 h. Cells were fixed and apoptosis was monitored by using a TUNEL assay. Where indicated, polyclonal anti-CCN1 antibodies were preincubated with test reagents for 30 min prior to addition into medium. Data shown are mean \pm S.D. of three determinations and representative of three experiments.

[0032] Fig. 16 demonstrates that CCN1 mutants may induce integrin $\alpha_v\beta_3$ -dependent endothelial tubule formation. HUVECs were either treated with vehicle buffer (no add) or stimulated with 5 nM PMA in serum-free medium before being plated on 24-well plates pre-coated with type I collagen gel (2 mg/ml) in the absence (-) or presence of CCN1 (WT) or TM (20 μ g/ml each). A second layer of gel of identical formulation was overlaid on the attached cells, and tubule formation was assessed 16 h thereafter. Where indicated, LM609 (40 μ g/ml) or GoH3 (40 μ g/ml) was added to cell suspensions prior to plating. Results are representative of three experiments (x 200 magnification).

DETAILED DESCRIPTION

[0033] CCN1 is an angiogenic inducer that plays an essential role in normal vascular development during embryogenesis. See Mo *et al.* (2002) *Mol. Cell Biol.* 22, 8709-8720. See also U.S. Pat. No. 6,413,735, and U.S. Application Serial No. 09/495,448 (allowed), both incorporated herein by reference. We have recently shown that the proangiogenic activities of CCN1 are mediated through integrins $\alpha_6\beta_1$ and $\alpha_v\beta_3$ in unactivated and activated HUVECs, respectively. See Leu *et al.* (2002) *J. Biol. Chem.* 277, 46248-46255. In addition to integrin $\alpha_6\beta_1$ interaction with the T1 sequence in the TSP1 domain of CCN1, adhesion of fibroblasts and unactivated endothelial cells to CCN1 also requires heparin sulfate proteoglycans to act as co-receptors which interact with the heparin binding motifs in the CCN1 C-terminal domain. See Chen *et al.* (2000) *J. Biol. Chem.* 275, 24953-24961. In this study, we have employed functional and biochemical analyses to define a 17-residue T1 sequence (GQKCIVQTTSWSQCSKS) in the

CCN1 domain III as a novel integrin $\alpha_6\beta_1$ binding site. We have also determined that heparin binding sites H1 and H2 are influential in the activity of the $\alpha_6\beta_1$ -HSPG coreceptor complex in functions such as cell adhesion that are an important part of angiogenesis. These findings provide a basis for the development of $\alpha_6\beta_1$ antagonists and a target for mutational analyses to examine the role of integrin $\alpha_6\beta_1$ -CCN1 interaction in angiogenesis.

Definitions

[0034] As used herein, the term "administer" means a single dose or multiple doses of a composition of the present invention.

[0035] As used herein, the term "treat" or "treating" when referring to protection of a mammal from a condition, means preventing, suppressing, repressing, or eliminating the condition. Preventing the condition involves administering a composition of the present invention to a mammal prior to onset of the condition. Suppressing the condition involves administering a composition of the present invention to a mammal after induction of the condition but before its clinical appearance. Repressing the condition involves administering a composition of the present invention to a mammal after clinical appearance of the condition such that the condition is reduced or maintained. Elimination of the condition involves administering a composition of the present invention to a mammal after clinical appearance of the condition such that the mammal no longer suffers the condition.

Cell Adhesion is modulated by the $\alpha_6\beta_1$ -HSPG coreceptor complex

[0036] Consistent with our earlier findings that a truncated mutant encompassing domains I-III of CCN1 is capable of inducing $\alpha_6\beta_1$ -dependent smooth muscle cell migration (*see* Grzeszkiewicz *et al.* (2002) *Endocrinology* 143, 1441-1450), we find that a recombinant fragment corresponding to the isolated domain III (TSP1 domain) of murine CCN1 is sufficient to support $\alpha_6\beta_1$ -dependent fibroblast adhesion. Along with $\alpha_6\beta_1$, heparin sulfate proteoglycans (HSPGs) operate as co-receptors of that interaction. *See* Chen, N., Chen, C. C., and Lau, L. F. (2000) *J. Biol. Chem.* 275, 24953-24961 and Leu, S.-J., Lam, S. C. T., and Lau, L. F. (2002) *J. Biol. Chem.* 277, 46248-46255. The specificity of $\alpha_6\beta_1$ interaction with the murine CCN1 domain III is confirmed by the failure of the murine CCN1 domain I and domain II fragments to support cell adhesion, and by the observation that anti- α_6 and anti- β_1 mAbs specifically block cell adhesion to the murine CCN1 domain III (Figs. 1 and 2).

[0037] Within domain III, we have further pin-pointed the T1 sequence as an integrin $\alpha_6\beta_1$ binding site in CCN1 based on the following observations: 1) a T1-GST fusion protein and a synthetic T1 peptide specifically support $\alpha_6\beta_1$ -dependent cell adhesion (Figs. 3 and 4); 2) integrin $\alpha_6\beta_1$ is purified from a detergent lysate of fibroblasts on a T1-GST affinity matrix, demonstrating direct interaction between integrin $\alpha_6\beta_1$ and the T1 sequence (Fig. 7); 3) soluble T1 peptide inhibits cell adhesion to $\alpha_6\beta_1$ ligands including CCN1, CCN2, CCN3 and laminin, but not to other integrin ligands such as fibronectin, vitronectin and collagen (Fig. 5); and 4) T1 peptide also blocks $\alpha_6\beta_1$ -dependent tubule formation of unactivated HUVECs in a collagen matrix containing CCN1 (Fig. 8). It is noteworthy that soluble T1 peptide is an effective inhibitor on $\alpha_6\beta_1$ -dependent cellular activities. Half maximal inhibition of cell adhesion occurs at a peptide concentration of 25-50 mM (Fig. 5C). Thus, the inhibitory potency of T1 is comparable to linear RGD peptides that inhibit adhesive functions of other integrins, such as $\alpha_v\beta_3$, also at the micromolar range. See Ruoslahti (1996) *Ann. Rev. Cell Dev. Biol.* 12, 697-715.

[0038] Inasmuch as the T1 site may be shown to affect the binding of CCN1 with $\alpha_6\beta_1$, we have further shown that heparin binding sites H1 and H2 account for the remainder of $\alpha_6\beta_1$ -dependent activities of CCN1. Upon preparation of full-length CCN1-GST fusion proteins having mutations at the T1 site and the H1 and H2 heparin binding sites, mutants disrupted at all three of the sites lost all ability to support $\alpha_6\beta_1$ -mediated fibroblast adhesion. As described in Examples 11-13, while mutants disrupted at the H1 and H2 sites could support fibroblast adhesion only at an elevated concentration, mutants disrupted only at the T1 site were still able to support fibroblast adhesion at a level near that of the wild type.

[0039] Heparin binding at H1 and H2 of CCN1 may also be shown to affect the sustained activation of p42/p44 MAPKs.

[0040] By alanine substitution mutagenesis of the T1-GST fusion protein, we showed that the C-terminal portion of T1 (TTSWSQCSKS) contains critical determinants for $\alpha_6\beta_1$ -dependent cell adhesion. Of note is the double T231A/T232A and W234A/K239A substitutions that result in complete loss of its capacity to support cell adhesion. This 10-residue segment is highly conserved among other CCN family members with only two non-conserved substitutions among CCN1, CCN2 and CCN3. Therefore, it is conceivable that $\alpha_6\beta_1$ also binds to the corresponding

T1 sequences in other CCN proteins. Consistent with this notion, soluble T1 peptide also inhibits $\alpha_6\beta_1$ -dependent fibroblast adhesion to CCN2 and CCN3. These results lead us to conclude that the conserved TTXWSXCSKS sequence (*X* represents a non-conserved residue) in CCN proteins defines a novel recognition motif for integrin $\alpha_6\beta_1$. An important feature of this sequence is that any single alanine substitution of the conserved residues (i.e., T232A, W234A, S235A, S238A, and K239A) results in a drastic loss of $\alpha_6\beta_1$ binding activity, suggesting that it requires multiple coordination interaction with the ligand binding pocket in integrin $\alpha_6\beta_1$.

[0041] Integrin $\alpha_6\beta_1$ has a limited ligand spectrum that includes laminin, CCN proteins, invasin, fertilin and a collagen fragment known as tumstatin. See Sonnenberg *et al.* 1990) *J. Cell Biol.* 110, 2145-2155; Maeshima *et al.* (2001) *J Biol.Chem.* 276, 15240-15248; Isberg *et al.* (1990) *Cell* 60, 861-871; Almeida *et al.* (1995) *Cell* 81, 1095-1104. These diverse $\alpha_6\beta_1$ ligands that are involved in various biological processes are not structurally related. Several $\alpha_6\beta_1$ binding sequences have been identified by screening synthetic peptides derived from some of these $\alpha_6\beta_1$ ligands. These include the NPWHSIYTTRFG and TWYKIAFQRNRK sequences from the laminin a1 chain. See Sonnenberg *et al.* 1990) *J. Cell Biol.* 110, 2145-2155; Nomizu *et al.* (1995) *J. Biol. Chem.* 270, 20583-20590; Nakahara *et al.* (1996) *J. Biol. Chem.* 271, 27221-27224. In addition, TDE-containing peptides from the disintegrin domain of the fertilin b subunit disrupt sperm-egg fusion presumably by blocking integrin $\alpha_6\beta_1$ -fertilin interaction. See Myles *et al.* (1994) *Proc. Natl. Acad. Sci. U.S.A.* 91, 4195-4198. Several other $\alpha_6\beta_1$ binding peptides have also been isolated by screening phage display and synthetic peptide combinatorial libraries; however, these sequences are not present in any known $\alpha_6\beta_1$ ligand. See Murayama *et al.* (1996) *J.Biochem.(Tokyo)* 120, 445-451; Pennington *et al.* (1996) *Mol.Divers.* 2, 19-28; DeRoock *et al.* (2001) *Cancer Res.* 61, 3308-3313. A comparison of the $\alpha_6\beta_1$ binding sequences reported to date reveals no consensus sequence that acts as an $\alpha_6\beta_1$ binding motif. Furthermore, our newly identified T1 sequence in CCN1 does not exhibit any sequence similarity to these $\alpha_6\beta_1$ binding peptides. Thus, integrin $\alpha_6\beta_1$, like $\alpha_M\beta_2$, is capable of recognizing a broad range of binding sequences. At present, whether these vastly different peptide sequences bind to the same or different sites in $\alpha_6\beta_1$ remains to be determined. Nonetheless, given that integrin $\alpha_6\beta_1$ has been implicated in a multitude of biological processes, different $\alpha_6\beta_1$ binding sequences may

interact with distinct coordination sites within the $\alpha_6\beta_1$ ligand binding pocket to induce different signaling pathways that mediate disparate biological activities.

[0042] To date, three CCN proteins have been shown to induce neovascularization *in vivo*. See Babic *et al.* (1998) *Proc. Natl. Acad. Sci. U.S.A.* **95**, 6355-6360; Lin *et al.* (2003) *J. Biol. Chem.* In press; Babic *et al.* (1999) *Mol. Cell. Biol.* **19**, 2958-2966; Shimo *et al.* (1999) *J. Biochem. (Tokyo.)* **126**, 137-145; Fataccioli *et al.* (2002) *Hum. Gene Ther.* **13**, 1461-1470. Endothelial cell migration, proliferation, and differentiation into tubule structures are essential for the formation of new blood vessels. CCN1 is an activation-independent ligand of integrin $\alpha_6\beta_1$ in non-stimulated endothelial cells, mediating both cell adhesion and tubule formation through this integrin receptor. See Leu *et al.* (2002) *J. Biol. Chem.* **277**, 46248-46255. Whereas intact CCN1 is an angiogenic inducer, the T1 peptide acts as an $\alpha_6\beta_1$ antagonist to block CCN1-induced tubule formation of unactivated endothelial cells. Interestingly, the T1 sequence resides within the thrombospondin type 1 repeat homology domain of CCN1, and thrombospondin is an inhibitor of angiogenesis with its anti-angiogenic activity being localized to the procollagen homology region and the properdin-like type 1 repeat. See Tolsma *et al.* (1993) *J. Cell Biol.* **122**, 497-511. A number of anti-angiogenic peptides have been derived from thrombospondin, including the CSVTCG-containing peptides that interact with CD36 on endothelial cells. See Jimenez *et al.* (2000) *Nat. Med.* **6**, 41-48; Dawson *et al.* (1997) *J. Cell Biol.* **138**, 707-717. Interaction of CD36 with the TSP1 domain of CCN proteins has not been demonstrated; however, CD36 has been shown to associate with integrin $\alpha_6\beta_1$ on human platelets and melanoma cells. Miao *et al.* (2001) *Blood* **97**, 1689-1696; Thorne *et al.* (2000) *J. Biol. Chem.* **275**, 35264-35275. If the CD36- $\alpha_6\beta_1$ complex also exists on endothelial cells, it is an intriguing possibility that these two cell surface receptors may act in concert to regulate angiogenesis through interaction with proximal recognition sequences in the thrombospondin type 1 repeat of matricellular proteins.

1. CCN1 Fragments

[0043] The present invention relates to peptide fragments of CCN1 that modulate the activity of CCN1. The peptides may be used in therapeutic strategies designed to inhibit or induce the activity of CCN1. The peptides may be natural, synthetic or recombinant. One approach is to

produce a peptide comprising a sequence selected from the group consisting of: amino acids 224-240 of murine CCN1, amino acids 231-240 of murine CCN1, amino acids 226-242 of human CCN1, and amino acids 233-242 of human CCN1. For example, a peptide comprising conserved amino acids may compete with native CCN1 for its binding sites on integrins and other proteins. This competition may thereby inhibit the action of native CCN1. The present invention also relates to fragments of the CCN1 fragments. The peptides may be from 8 to 50 amino acids in length. The inhibitory peptides may be 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49 or 50 amino acids in length.

[0044] The peptides may also be homologs of the above-described CCN1 peptides. Homologs of the CCN1 peptides are peptides sharing a common evolutionary with CCN1. The peptides may also be variants of the above-described CCN1 peptides and homologs. peptide variants are peptides that differ in amino acid sequence from a native CCN1 peptide by the insertion, deletion, or conservative substitution of amino acids, but retain at least one biological activity of a native CCN1 peptide. For purposes of the present invention, "biological activity of a CCN1 peptide" includes, but is not limited to, the above-described activities of full-length CCN1, the ability to modulate activities of CCN1 and the ability to be bound by an antibody specific for CCN1.

[0045] A conservative substitution of an amino acid, *i.e.*, replacing an amino acid with a different amino acid of similar properties (*e.g.*, hydrophilicity, degree and distribution of charged regions) is recognized in the art as typically involving a minor change. These minor changes can be identified, in part, by considering the hydropathic index of amino acids, as understood in the art. *Kyte et al., J. Mol. Biol.* 157:105-132 (1982). A listing of the hydropathic indices of amino acids may be found in U.S. Pat. No. 6,639,054, which is incorporated herein by reference. It is known in the art that amino acids of similar hydropathic indexes can be substituted and still retain protein function. In one aspect, amino acids having hydropathic indexes of ± 2 are substituted.

[0046] The hydrophilicity of amino acids can also be used to reveal substitutions that would result in proteins retaining biological function. A consideration of the hydrophilicity of amino acids in the context of a peptide permits calculation of the greatest local average hydrophilicity

of that peptide, a useful measure that has been reported to correlate well with antigenicity and immunogenicity. A listing of hydrophilicity indices of amino acids may be found in U.S. Patent No. 4,554,101, which is incorporated herein by reference. Substitution of amino acids having similar hydrophilicity values can result in peptides retaining biological activity, for example immunogenicity, as is understood in the art. In one aspect, substitutions are performed with amino acids having hydrophilicity values within ± 2 of each other. Both the hydrophobicity index and the hydrophilicity value of amino acids are influenced by the particular side chain of that amino acid. Consistent with that observation, amino acid substitutions that are compatible with biological function are understood to depend on the relative similarity of the amino acids, and particularly the side chains of those amino acids, as revealed by the hydrophobicity, hydrophilicity, charge, size, and other properties.

[0047] Additionally, computerized algorithms are available to assist in predicting amino acid sequence domains likely to be accessible to an aqueous solvent. These domains are known in the art to frequently be disposed towards the exterior of a peptide, thereby potentially contributing to binding determinants, including antigenic determinants.

[0048] The peptides may also be analogs of the above-described CCN1 peptides, homologs and variants comprising non-standard amino acid or other structural variation from the conventional set of amino acids. The peptides may also be derivatives of the above-described CCN1 peptides, homologs, variants and analogs that differ in ways other than primary structure (amino acids and amino acid analogs). By way of illustration, derivatives may differ from native CCN1 peptides, homologs and variants by being glycosylated, one form of post-translational modification. For example, polypeptides may exhibit glycosylation patterns due to expression in heterologous systems. If these peptides retain at least one biological activity of native CCN1, then these peptides are CCN1 derivatives according to the invention. Other derivatives include, but are not limited to, fusion peptides having a covalently modified N- or C-terminus, PEGylated peptides, peptides associated with lipid moieties, alkylated peptides, peptides linked via an amino acid side-chain functional group to other peptides or chemicals, and additional modifications as would be understood in the art. In addition, the invention contemplates CCN1-related peptides that bind to a CCN1 receptor, as described below.

[0049] The various peptides of the present invention, as described above, may be provided as discrete peptides or be linked, *e.g.*, by covalent bonds, to other compounds. For example, immunogenic carriers such as Keyhole Limpet Hemocyanin may be bound to a CCN1 peptide of the invention. The various fragments, variants, analogs, homologs or derivatives described above may be 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, or 99% identical to the peptides. The present invention is also related to isolated nucleic acids encoding the peptides. The present invention also involves a pharmaceutical composition comprising peptides of the present invention.

2. Antibodies

[0050] The present invention also involves a pharmaceutical composition comprising an antibody that specifically binds to the CCN1 peptides of the present invention and a pharmaceutically acceptable adjuvant, diluent, or carrier. The antibody may be produced as described below, or as described in WO 01/55210, the contents of which are hereby incorporated by reference in their entirety.

[0051] The antibodies of the present invention include antibodies of classes IgG, IgM, IgA, IgD, and IgE, and fragments and derivatives thereof including Fab and F(ab')₂. The antibodies may also be recombinant antibody products including, but not limited to, single chain antibodies, chimeric antibody products, "humanized" antibody products, and CDR-grafted antibody products. The antibodies of the present invention include monoclonal antibodies, polyclonal antibodies, affinity purified antibodies, or mixtures thereof which exhibit sufficient binding specificity to the CCN1 fragments.

[0052] Also contemplated by the invention are antibody fragments. The antibody products include the aforementioned types of antibody products used as isolated antibodies or as antibodies attached to labels. Labels can be signal-generating enzymes, antigens, other antibodies, lectins, carbohydrates, biotin, avidin, radioisotopes, toxins, heavy metals, and other compositions known in the art; attachment techniques are also well known in the art.

[0053] Anti-CCN1 antibodies are useful in diagnosing the risk of oncogenesis. In addition, anti-CCN1 antibodies may be used in therapies designed to deliver specifically-targeted cytotoxins to cells expressing CCN1, *e.g.*, cells participating in the neovascularization of solid tumors. These

antibodies are delivered by a variety of administrative routes, in pharmaceutical compositions comprising carriers or diluents, as would be understood by one of skill in the art.

3. Screening for Modulators of CCN1

[0054] The present invention involves screening for modulators of activities associated with CCN1. Modulators may be identified that interact with the integrin binding site of CCN1, thereby preventing CCN1 from interacting with target integrins and other proteins. Modulators may also be identified that directly bind to targets integrins and other proteins of CCN1, thereby preventing CCN1 from productively interacting with said target integrins and other proteins. Modulators may also be identified which indirectly affect binding of CCN1 to target proteins.

[0055] For purposes of the invention, an "ECM signaling molecule" refers to a CCN1 fragment described above. The use of "ECM signaling molecule" also contemplates one or more additional CCN polypeptides. The one or more additional CCN polypeptides include, but are not limited to, CCN1, CCN2, CCN4, CCN5 and CCN6, as well as fragments, variants, analogs, homologs or derivatives of said one or more additional CCN polypeptides.

a. Angiogenesis

[0056] The methods of the present invention relate to screening for a modulator of angiogenesis. In one embodiment of the present invention, a biological sample capable of undergoing angiogenesis is contacted with a suspected modulator *in vitro* along with an ECM signaling molecule. As a control, a second biological sample is also contacted with an ECM signaling molecule. A modulator of angiogenesis may be identified by its ability to alter the level of angiogenesis of the test sample when compared to the control sample.

[0057] In another embodiment of the present invention, an implant comprising a suspected modulator and an ECM signaling molecule is implanted into a test animal. As a control, a second implant comprising an ECM signaling molecule is implanted into a test animal, which may be the same animal or a different test animal. A modulator of angiogenesis may be identified by its ability to alter the level of blood vessel development in the test implant when compared to the control sample.

b. Oncogenesis

[0058] The methods of the present invention also relate to screening for a modulator of oncogenesis. A tumor is contacted with a suspected modulator along with an ECM signaling molecule. As a control, a second tumor is also contacted with an ECM signaling molecule. A modulator of oncogenesis may be identified by its ability to alter the level of oncogenesis of the test tumor when compared to the control tumor.

c. Cell Adhesion

[0059] The methods of the present invention also relate to screening for a modulator of cell adhesion. A biological sample on a surface compatible with cell adherence is contacted with a suspected modulator along with an ECM signaling molecule. As a control, a second biological sample on a surface compatible with cell adherence is also contacted with an ECM signaling molecule. A modulator of cell adhesion may be identified by its ability to alter the level of cell adhesion of the test sample when compared to the control sample.

d. Cell Migration

[0060] The methods of the present invention also relate to screening for a modulator of cell migration. Cells capable of undergoing cell migration are seeded onto a gel matrix comprising a suspected modulator and an ECM signaling molecule. As a control, cells capable of undergoing cell migration are also seeded onto a second biological sample gel matrix comprising an ECM signaling molecule. A modulator of cell adhesion may be identified by its ability to alter the level of cell migration in the test matrix when compared to the control matrix.

e. Methods of Treatment

[0061] The present invention also involves modulators of CCN1 activity identified using the above-described screening methods. The identified modulators of CCN1 activity may be formulated in a pharmaceutical composition comprising a pharmaceutically acceptable adjuvant, diluent, or carrier. The pharmaceutical composition comprising the modulator of CCN1 activity may be administered to a patient for the treatment of disease associated with angiogenesis, oncogenesis, or chondrogenesis. The pharmaceutical composition may be administered alone or in combination with other compositions, such as a chemotherapeutic.

[0062] Compositions of the present invention may be administered in a standard manner including, but not limited to, orally, parenterally, sublingually, transdermally, rectally, transmucosally, topically, via inhalation, or via buccal administration. Parenteral administration includes, but is not limited to, intravenous, intraarterial, intraperitoneal, subcutaneous, intramuscular, intrathecal, and intraarticular.

[0063] Having now generally described the invention, other aspects and advantages of the present invention will be understood upon consideration of the following illustrative examples. These examples are for purposes of illustration only and are not intended to limit the scope of the invention as set out in the appended claims. Example 1 discloses that domain III of CCN1 supports $\alpha_6\beta_1$ -dependent cell adhesion. Example 2 discloses that the T1 sequence in domain III of CCN contains an integrin $\alpha_6\beta_1$ binding site. Example 3 describes the effect of soluble T1 peptide on $\alpha_6\beta_1$ -dependent cell adhesion. Example 4 describes the effect of alanine substitutions in the T1 sequence on cell adhesion. Example 5 describes affinity purification of integrin $\alpha_6\beta_1$ using a T1-coupled affinity matrix. Example 6 describes the effect of soluble T1 peptide on CCN1-induced endothelial tubule formation. Example 7 describes the construction of mutant peptides inactivated at T1 binding site for integrin $\alpha_6\beta_1$. Example 8 describes evaluation of adhesion of mutant CCN1 peptide inactivated at T1 binding site for integrin $\alpha_6\beta_1$. Example 9 describes construction of mutant CCN1 inactivated at T1 binding site for integrin $\alpha_6\beta_1$. Examples 10-14 describe cell adhesive properties of CCN1 mutants. Example 10 describes an SM assay using a disrupted T1 binding site with an uninterrupted heparin binding site. Example 11 describes an SM Assay using a disrupted T1 binding site, with inhibition of heparin binding sites. Example 12 describes a DM assay using an uninterrupted T1 binding site with a disrupted heparin binding site. Example 13 describes a TM assay using a disrupted T1 binding site, and a disrupted heparin binding site. Example 14 describes the use of anti-integrins to evaluate cell adhesion of DM. Examples 15-16 describe the effect of disruption of H1 and H2 on CCN1-dependent MAPK activation and regulation of gene expression. Example 15 describes MAPK activation. Example 16 describes regulation of gene expression. Examples 17-21 describe the dissociation of CCN1 activities mediated through $\alpha_v\beta_3$ and $\alpha_6\beta_1$ -HSPGs. Example 17 describes HUVEC adhesion and migration to CCN1 mutants through integrin $\alpha_v\beta_3$. Example 18 describes migration of HUVECS to CCN1 or mutants. Example 19 describes enhancement of VEGF-induced DNA Synthesis through integrin $\alpha_v\beta_3$. Example 20 describes the effect of CCN1

mutants on HUVEC survival. Example 21 describes tubule formation. These examples are intended to be illustrative of the present invention and should not be construed to limit the scope of the invention.

EXAMPLE 1

Domain III (TSP1-homology Domain) of CCN1 Supports $\alpha_6\beta_1$ -dependent Cell Adhesion

[0064] Previous studies have established that primary human skin fibroblasts adhere to CCN1 through integrin $\alpha_6\beta_1$ and heparin sulfate proteoglycans, inducing the formation of $\alpha_6\beta_1$ -containing focal complexes and the activation of focal adhesion kinase, paxillin, and Rac. *See* Chen *et al.* (2000) *J.Biol.Chem.* **275**, 24953-24961; Chen *et al.* (2001) *J.Biol.Chem.* **276**, 10443-10452. Deletion analysis showed that a C-terminal truncated CCN1 mutant containing only the first three domains retains the ability to induce chemotaxis in smooth muscle cells through integrin $\alpha_6\beta_1$, thus localizing an integrin $\alpha_6\beta_1$ binding site within the first three domains. *See* Grzeszkiewicz *et al.* (2002) *Endocrinology* **143**, 1441-1450.

[0065] To define the CCN1 structural domain that interacts with integrin $\alpha_6\beta_1$, we expressed each of these three domains in insect cells via a baculovirus vector (Fig. 1A). To enhance protein secretion, we employed the pMelBac B vector (Invitrogen Incorp.) to provide an N-terminal honeybee melittin secretion signal peptide. To produce the coding sequences for domain I (IGFBP), domain II (VWC), and domain III (TSP1), we used the following respective primer sets for PCR upon the CCN1 cDNA:

5'-CGCGGATCCGGCGCTCTCCACCTGC-3' and

5'-GGAATTCCCTCTGCAGATCCCTTTCAGAGCGG-3'

5'-CGCGGATCCGGCTCAGTCAGAAGGCAGAC-3' and

5'-GGAATTCCCAGGAAGCCTCTTCAGTGAGCTGCC-3'

5'-CGCGGATCCGGTCTTTGGCACC-3' and

5'-GGAATTCCTTTTAGGCTGCTGTACACTGGTTGTC-3'

[0066] The PCR products were digested with *Bam*H1 and *Eco*R1, and ligated into pMelBac B. Each expressed recombinant polypeptide contained the V5 epitope and a polyhistidine tag at the C-terminus, and was purified from Sf9 cells using a serum-free baculovirus expression system as described. See Grzeszkiewicz *et al.* (2001) *J Biol. Chem.* 276, 21943-21950.

[0067] Briefly, cells were maintained under serum-free conditions in EX-CELL 400 medium (JRH Biosciences, Lenexa, KS), infected at a multiplicity of infection of 10, and collected 42~46 h post-infection. The collected medium was cleared by centrifuge and subsequently concentrated by 10~15 folds using the Biomax-5 centrifugal filter (Millipore, Billerica, MA), and dialyzed against native buffer (50 mM sodium phosphate and 10 mM Hepes at pH 7.4, 0.5 M NaCl) overnight at 4°C and then applied to a TALON cobalt-agarose column (Clontech, Palo Alto, CA). The column was washed with native binding buffer at pH 7.0, before being eluted in 50 mM phosphate at pH 7.0, 0.3 M NaCl, and 150 mM imidazole. Products were analyzed by SDS-PAGE followed by Coomassie Brilliant Blue staining and immunoblotting. The pooled fractions for each domain fragment were dialyzed against 20 mM Hepes at pH 7.4, 150 mM NaCl overnight at 4°C to remove imidazole. Each domain fragment had the expected molecular mass (~11 kDa, 18 kDa, and 9 kDa for domains I, II, and III, respectively), and were immunoreactive with an anti-CCN1 polyclonal antibody (Fig. 1B and C).

[0068] We employed human 1064SK fibroblasts to address the ability of each domain to support cell adhesion. Primary human foreskin fibroblast 1064SK (ATCC CRL-2076, passage 2) were kept in Iscove's modified Dulbecco's medium (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (Intergen, Purchase, NY) at 37°C with 5% CO₂. Cells were used within the 5th to 20th passages for all experiments. Test proteins were coated onto 96-well microtiter plates (Becton Dickinson, NJ) in PBS (50 ml per well), and wells were blocked with 1% BSA (Sigma, St. Louis, MO) at room temperature for 1 h. To enhance coating efficiency, CCN1 domain polypeptides were covalently linked to maleic anhydride Reacti-Bind microtiter plates (Pierce, Rockford, IL) at 4°C overnight followed by blocking with BSA at 37°C for 2 h. Cell adhesion was conducted using washed subconfluent cells resuspended in serum-free basal medium at 5 x 10⁵ cells/ml as described. See Chen *et al.* (2001) *J.Biol.Chem.* 276, 10443-10452. Where

indicated, cells were preincubated with EDTA, peptides, or function-blocking mAbs for 30 min prior to plating.

[0069] All three domains were coated onto microtiter wells with similar efficiency. Microtiter wells were coated with hexahistadine-tagged proteins or BSA (50 µl/well) overnight at 4°C, followed by blocking with 1% BSA for 2 h at room temperature. Protein coating efficiency was examined by incubation with an anti-polyhistidine mAb (Invitrogen) (2 h at 37°C) followed by an horseradish peroxidase-conjugated secondary antibody (Amersham Pharmacia Biotech-Piscataway, NJ) (1 h at 37°C). The color reaction was developed and quantified by A₄₂₀ measurements.

[0070] Only Domain III was able to support fibroblast adhesion (Fig. 1D and E). Fibroblast adhesion to Domain III was inhibited by EDTA (2.5 mM), and this inhibition was relieved by the addition of Mg⁺⁺ (5 mM) in the assay media (Fig. 2A). Cell adhesion was also inhibited by Ca⁺⁺ (5 mM) and promoted by Mn⁺⁺ (0.5 mM). This divalent-cation sensitivity profile is similar to that of full-length CCN1, and is consistent with cell adhesion through integrin α₆β₁. *See* Chen *et al.* (2000) *J.Biol.Chem.* 275, 24953-24961. To ascertain which specific integrin receptor mediated cell adhesion to Domain III, we tested the inhibitory attributes of function-blocking mAbs. Preincubation of fibroblasts with mAbs against α₆ (GoH3) or β₁ (P4C10) obliterated cell adhesion to Domain III as well as full-length CCN1, whereas mAb against integrin α_vβ₃ (LM609) or control mouse IgG had no effect (Fig. 2B). Together, these results show that human skin fibroblasts adhesion to the isolated Domain III of CCN1, like adhesion to full-length CCN1, is mediated through integrin α₆β₁.

EXAMPLE 2

The T1 Sequence in Domain III of CCN1 Contains an Integrin α₆β₁ Binding Site

[0071] We employed another systematic screening strategy to pinpoint the integrin α₆β₁ binding site in CCN1. A series of overlapping peptides (Table 1) that covers the entire first three domains of CCN1 was prepared by expression of the peptides as fusion proteins linked to GST. The coding sequences for various peptides (Fig. 3) were amplified by polymerase chain reactions (PCR) upon the CCN1 cDNA as template. Primers used corresponded to the appropriate coding

sequences and contain the *Bam*HI and *Eco*RI restriction sites for cloning. For example, the following primers were used to generate the T1 peptide coding sequence:

5'-CGGGATCCGCGGGGCCAGAAATGCATCGTT-3' and

5'-CCGGAATTCCGCTCTTGGAGCACTGGGACC-3'

PCR products were purified on polyacrylamide gels, digested with *Bam*HI and *Eco*RI, and ligated into the pGEX-4T-2 vector (Amersham Pharmacia Biotech). All cloning steps were confirmed by sequence analysis. GST-peptide fusion proteins were produced in *E. coli* strain BL21 and purified by glutathione affinity chromatography (Amersham Pharmacia Biotech), followed by extensive dialysis against PBS overnight at 4°C.

[0072] These fusion proteins were purified to near homogeneity, and had similar levels of coating efficiency in microtiter wells as detected by ELISA using an anti-GST antibody (data not shown). The ability of each peptide-GST fusion protein to support fibroblast adhesion was assessed. Only one peptide-fusion protein, namely T1 from Domain III, was able to support cell adhesion (Fig. 3A). Again, fibroblast adhesion to T1-GST was inhibited by EDTA and Ca^{++} , and promoted by Mn^{++} in the assay media (Fig. 3B). Also, cell adhesion to T1-GST was blocked by preincubation of cells with anti- α_6 (GoH3) or anti- β_1 (P4C10) mAb, but unaffected by other integrin-disrupting agents such as GRGDSP peptide (Life Technologies/Gibco-BRL) or anti- $\alpha_v\beta_3$ (LM609) (Fig. 3C), indicating that the T1-GST fusion protein supports the adhesion of fibroblasts through integrin $\alpha_6\beta_1$. Likewise, T1-GST also supported $\alpha_6\beta_1$ -mediated cell adhesion in other cell types, including endothelial cells, smooth muscle cells, and PC3 prostate cancer cells (data not shown).

[0073] To establish further that the T1 sequence contains a binding site for integrin $\alpha_6\beta_1$, four peptides spanning the CCN1 domain III (Table 1) were synthesized and tested for the abilities to support cell adhesion. The synthetic peptides were prepared by ResGen Inc. (Huntsville, AL), followed by purification on reverse-phase high performance liquid chromatography and analysis by mass spectroscopy. Similar to the results obtained with GST-peptide fusion proteins, synthetic T1 peptide, but not the other 3 peptides (T2, T3 and T4), supported fibroblast adhesion

(Fig. 4A). Moreover, cell adhesion to immobilized T1 peptide was inhibited by anti- α_6 (GoH3) or anti- β_1 (P4C10), but not by anti- $\alpha_v\beta_3$ (LM609) or control mouse IgG (Fig. 4B). These results again indicate that T1 contains an integrin $\alpha_6\beta_1$ binding site.

EXAMPLE 3

Soluble T1 peptide Inhibits $\alpha_6\beta_1$ -dependent Cell Adhesion

[0074] We anticipated that soluble T1 peptide is capable of blocking cell adhesion to substrates known to bind integrin $\alpha_6\beta_1$. As shown in Fig. 5A, addition of 0.2 mM T1 to the cell suspension effectively blocked fibroblast adhesion to CCN1, whereas T2, T3, or T4 had no effect. The inhibitory effect of T1 on cell adhesion to CCN1 was dose-dependent, achieving maximal inhibition at 100 μ M (Fig. 5C). Other members of the CCN protein family, CCN2 (CTGF) and CCN3 (NOV), have also been shown to support fibroblast adhesion through integrin $\alpha_6\beta_1$ (See Chen *et al.* (2001) *J.Biol.Chem.* **276**, 10443-10452; Lin *et al.* (2003) *J.Biol.Chem.* In press), and a high degree of homology exists among the corresponding T1 sequences in these CCN proteins. Fig. 5A shows that T1 also specifically inhibited cell adhesion to CCN2 and CCN3, suggesting that the T1 sequence in CCN proteins is a common binding site for integrin $\alpha_6\beta_1$.

[0075] To demonstrate further the specificity of T1 inhibition, we examined its ability to block cell adhesion to substrates that bind other integrins. In contrast to its dose-dependent inhibitory effect of cell adhesion to CCN1 (Fig. 5C), T1 had no significant effect on the adhesion of fibroblasts to fibronectin (ligand of integrin $\alpha_5\beta_1$), vitronectin (ligand of α_v integrins), and collagen (ligand of β_1 -integrins) (Fig. 5B). Cell adhesion to laminin, a known ligand for integrin $\alpha_6\beta_1$, was partially inhibited by the T1 peptide (~15 %). This partial inhibition was similar to that achieved by the anti- α_6 mAb GoH3 (data not shown). Incomplete inhibition by T1 and GoH3 was likely due to the presence of other integrins, such as $\alpha_2\beta_1$, that also serve as adhesion receptors for laminin. Together, these results show that the soluble T1 peptide specifically inhibits $\alpha_6\beta_1$ -dependent cell adhesion, thus providing further support that the T1 sequence contains a binding site for integrin $\alpha_6\beta_1$.

EXAMPLE 4**Effect of Alanine Substitutions in the T1 Sequence on Cell Adhesion**

[0076] To determine which residues within the T1 sequence are critical determinants for $\alpha_6\beta_1$ -dependent cell adhesion, we prepared a series of GST-peptide fusions that carries the T1 backbone with single or double alanine substitutions at residues conserved among CCN1, CCN2, and CCN3, and tested their abilities to support cell adhesion. To generate site-directed alanine substitutions for the T1 peptide (Fig. 6), synthetic oligonucleotides were annealed for PCR to generate the appropriate coding sequences and cloned into pGEX-4T-2. The following primers were used to prepare the coding sequence for the T1 sequence and cloned into pGEX-4T-2:

5'-GATCCGGTCAAAAATGTATTGTTCAAACACTTCTTGG
TCTCAATGCTCTAAATCTGG-3' and

5'-AATTCCAGATTTAGAGCATTGAGACCAAGAAGTAGTA
GTTTGAACAATACATTTTGTACCG-3'

To create the mutant peptides, relevant codons were changed to either GCA or GCT for alanine.

[0077] As shown in Fig. 6, alanine substitutions at residues K226, I228, or Q230 did not affect the peptide's ability to support cell adhesion. While single mutation at either T231 or T232 resulted in partial reduction of cell adhesion, combined alanine substitutions of T231 and T232 completely abolished the ability of T1 to support cell adhesion. In addition, single substitutions in W234, S235, S238, or K239 resulted in >90% loss of T1 activity. When mutations in W234 and K239 were combined, cell adhesion was completely obliterated. These results indicate that TTSWSQCSKS is the core sequence in T1 for mediating $\alpha_6\beta_1$ binding. These data also explain the inability of the T2 peptide, which overlaps with the T1 peptide but lacks the TT residues of the core sequence, to inhibit $\alpha_6\beta_1$ -dependent cell adhesion.

EXAMPLE 5

Affinity Purification of Integrin $\alpha_6\beta_1$ Using a T1-coupled Affinity Matrix

[0078] To confirm that the T1 peptide binds directly to integrin $\alpha_6\beta_1$, we performed affinity chromatography using cell surface proteins on fibroblasts to isolate integrin $\alpha_6\beta_1$ on a T1-coupled affinity column. Subconfluent 1064SK fibroblasts were detached with 2 mM EDTA and 0.05% BSA in PBS, washed thrice and resuspended in PBS containing 20 mM glucose at 2×10^7 cells/ml. For surfacing labeling, the cell suspension was incubated with 100 mU/ml glucose oxidase, 200 mg/ml lactoperoxidase (Calbiochem-Novabiochem, La Jolla, CA), and ~400 mCi/ml carrier-free Na^{125}I (Amersham Pharmacia Biotech) for 30-60 min at 4°C with gentle rotation. To terminate labeling, 10 ml cell culture medium was added. The labeled cells were washed and solubilized in 1 ml of lysis buffer (50 mM Hepes, pH 7.4, 200 mM octyl-b-D-glucopyranoside, proteinase inhibitor cocktail, and 0.5 mM Mn^{++}). For affinity chromatography, GST-T1 or GST-scrambled T1 protein was coupled to Affi-Gel 10 (Bio-Rad Laboratories, Hercules, CA) at 10 mg/ml gel suspension. The labeled cell lysates were applied onto the affinity matrices (3 ml/ml gel) and incubated for 2 h at 4°C . The columns were washed with 30 column volumes of lysis buffer followed by elution with 0.35 M NaCl in the lysis buffer. The labeled proteins in the eluted fractions were analyzed by electrophoresis on 7% polyacrylamide gels under non-reducing conditions followed by autoradiography. In immunoprecipitation analyses, labeled proteins were incubated with 5 mg anti- α_6 (GoH3) or anti- α_v (P3G8) mAbs (Chemicon, Temecula, CA) as indicated. The immunoprecipitated proteins were collected on protein G-Sepharose and resolved on 7% polyacrylamide gels under non-reducing conditions.

[0079] A control column was prepared using GST fused to a scrambled T1 sequence, and no labeled protein band was eluted from the scrambled T1-GST column (Fig. 7A). By contrast, from the T1-GST affinity column, two protein bands with apparent molecular weights corresponding to integrin α_6 (~150 kDa) and β_1 (~130 kDa) subunits were eluted at 0.35 M NaCl (lanes 5-7, Fig. 7B). To confirm that the bound labeled proteins was indeed the integrin $\alpha_6\beta_1$ complex, the eluates were subjected to immunoprecipitation using GoH3 (anti- α_6) or P3G8 (anti- α_v) as a control. Fig. 7C shows that GoH3 immunoprecipitated the labeled protein bands from the eluate, whereas P3G8 failed to pull down the protein complex in the control sample. Collectively, we conclude that integrin $\alpha_6\beta_1$ binds directly to the T1 sequence in CCN1.

EXAMPLE 6**Soluble T1 peptide Disrupts CCN1-induced Endothelial Tubule Formation**

[0080] Several CCN proteins including CCN1, CCN2, and CCN3 are potent angiogenic inducers. *See Babic et al. (1998) Proc. Natl. Acad. Sci. U.S.A. 95, 6355-6360; Lin et al. (2003) J. Biol. Chem. In press; Babic et al. (1999) Mol. Cell. Biol. 19, 2958-2966.* Furthermore, when formulated into collagen gel, CCN1 is capable of inducing tubule formation of unactivated human umbilical vein endothelial cells (HUVECs), and this process is blocked by the anti- α_6 mAb GoH3. *See Leu et al. (2002) J. Biol. Chem. 277, 46248-46255.* Since the T1 sequence represents a major binding site for integrin $\alpha_6\beta_1$ in CCN1, we examined whether soluble T1 peptide would inhibit CCN1-induced tubule formation of unactivated HUVECs. To assay for endothelial cell tubule formation, human umbilical vein endothelial cells (HUVECs) were examined in a three-dimensional collagen gel in the presence or absence of test proteins or peptides as described. *See Leu et al. (2002) J. Biol. Chem. 277, 46248-46255.*

As shown in Fig. 8, when collagen gels are formulated with CCN1, human umbilical vein endothelial cells are induced to form tubules. Preincubation of HUVECs with T1 (0.2 mM) for 30 min prior to plating completely inhibited CCN1-induced tubule formation. By contrast, the control T2, T3 and T4 peptides had no effect. Together, these results indicate that T1 inhibits CCN1-induced tubule formation by blocking the interaction of CCN1 with integrin $\alpha_6\beta_1$ on unactivated HUVECs.

EXAMPLE 7**Construction of mutant peptides inactivated at T1 binding site for integrin $\alpha_6\beta_1$**

[0081] Several CCN proteins including CCN1, CCN2, and CCN3 are potent angiogenic. While useful in the determination of a novel $\alpha_6\beta_1$ binding site, the alanine-substituted GST-T1 fusion peptides described in Example 4 proved unsuitable for determination of the functional role of the T1 site in CCN1 activities. The expressed mutants aggregated and formed inclusion bodies inside the cell (data not shown), suggesting that these mutants adopted detrimental conformations that prevented them from being secreted. To create mutants specifically

inactivated in $\alpha_6\beta_1$ -HSPG-mediated activities while preserving other functions, we constructed fusion proteins using two algorithms, PSIPRED and FRAGFOLD (McGuffin, L. J., Bryson, K., and Jones, D. T. (2000) *Bioinformatics*. 16, 404-405, and Jones, D. T. (2001) *Proteins Suppl* 5, 127-132) to predict possible changes in secondary structures that could result from possible mutations.

[0082] We prepared GST fusion proteins of CCN1 peptides and mutant peptides according to methods described in Leu, S. J., Liu, Y., Chen, N., Chen, C. C., Lam, S. C., and Lau, L. F. (2003) *J. Biol. Chem.* 278, 33801-33808.

[0083] Mouse CCN1 engineered with a C-terminal FLAG tag was constructed by using the primer sets upon the mouse *Ccn1* cDNA as template

F1: 5'-CGCAATTGGAAAAGGCAGCTCACTGAAGAGGC-3' and

F2: 5'-CCGGAATTCCTACTTGTCATCGTCATCCTTGTAGTCGTCCCTGAA
CTTGTGGATGTCATTG-3',

thus yielding a PCR product containing the last codon of *Ccn1* followed by the FLAG tag coding sequence and a stop codon. The PCR product was double digested with *NcoI* and *EcoRI*, and ligated into pre-cut vector to substitute the *NcoI*, *EcoRI*-digested fragment of the full-length mouse *Ccn1* cDNA in pBlueBac4.5 vector (Grzeszkiewicz, T. M., Kirschling, D. J., Chen, N., and Lau, L. F. (2001) *J. Biol. Chem.* 276, 21943-21950). For consistency, WT CCN1 and all mutants used in this study were similarly constructed with the same N-terminal secretory signal and C-terminal FLAG epitope tag.

[0084] Consistent with the inability of alanine substitution mutants to be secreted, the T1 region of CCN1 appeared highly sensitive to perturbation. Substitutions in the critical T1 residues

(W234, S235, S238, K239) with nearly any amino acid resulted in drastic changes in the predicted protein structure. However, we found one mutation, namely K239E, that did not elicit a predicted conformational change. To test the efficacy of this mutation in disrupting interaction with $\alpha_6\beta_1$, we constructed a fusion protein with GST linked to the T1 peptide where the T1 peptide carried the K239E mutation (GQKCIVATTWSQCSES).

EXAMPLE 8

Evaluation of adhesion of mutant CCN1 peptide inactivated at T1 binding site for integrin $\alpha_6\beta_1$

[0085] We assessed fibroblast adhesion to the T1(K239E) mutant peptide constructed according to Example 7 as compared to GST-T1 fusion with the WT sequence. GST-T1 and GST-T1(K239E) have similar coating efficiency as determined by ELISA (data not shown).

[0086] We plated fibroblasts on microtiter wells coated with either GST, GST-T1 peptide fusion, or GST-T1(K239E) peptide fusion protein (50 $\mu\text{g}/\text{ml}$ each). Cells were allowed to adhere at 37°C for 20 min. After washing, adherent cells were fixed, stained with methylene blue, and quantified by absorbance at 620 nm.

[0087] As expected, fibroblasts adhered to the GST-T1 fusion but not to BSA nor the GST control (Fig. 9B). *See also* Leu, S. J., Liu, Y., Chen, N., Chen, C. C., Lam, S. C., and Lau, L. F. (2003) *J. Biol. Chem.* 278, 33801-33808. GST-T1(K239E) was completely unable to support cell adhesion, indicating that the charge-reversed K239E mutation may be sufficient to abolish T1 binding to integrin $\alpha_6\beta_1$.

EXAMPLE 9

Construction of mutant CCN1 inactivated at T1 binding site for integrin $\alpha_6\beta_1$

[0088] We constructed the K239E single mutation in the context of full length CCN1, designated SM (Fig. 9A) using site-directed mutagenesis using a two-step PCR procedure as described in Koskinen, P., Lehtvaslaiho, H., MacDonald Bravo, H., Alitalo, K., and Bravo, R. (1990) *Oncogene*. 5, 615-618. The internal primer sets were

5'-GTCTTGGTCCAGTGTTCCGAGAGCTGCGG-3' and

5'-CACTGGGACCAAGACGTGGTCTGAACGATGC-3'.

This construct also created a silent mutation at C237, thereby providing a screening marker by eliminating a BSP12861 restriction site. The outside primers used in PCR were F1 and F2 as described in Example A₁ above. SM was constructed using the mouse *Ccn1* cDNA with a FLAG tag as PCR template.

[0089] We also constructed the DM coding sequence, which has disrupted heparin binding sites H1 and H2 as shown in Fig. 9A. These mutations changed H1 from KGKKCSKTKKSPEPVR to AGAACSATAKSPEPVR and H2 from FTYAGCSSVKKYRPKY to FTYAGCSSVAAYAPKY, in the same background as CCN1. We used the DM coding sequence to generate the DM construct with a FLAG tag using the procedure described above. We created the TM sequence using DM as a template to generate the K239E mutation using the methods described above. All constructs were confirmed by direct sequence analysis.

EXAMPLE 10

SM Assay-Disrupted T1 binding site, uninterrupted heparin binding sites

[0090] We plated 1064SK human fibroblasts on microtiter wells coated with the indicated amounts of recombinant WT CCN1 or SM mutant, and assessed cell adhesion as described in Example 1. Surprisingly, SM was able to support fibroblast adhesion in a dose-dependent manner much like WT CCN1, achieving maximal cell adhesion at a coating concentration of 1 $\mu\text{g/ml}$ (Fig. 10A). This result demonstrates that SM may still support cell adhesion through the $\alpha_6\beta_1$ -HSPGs coreceptors, indicating that there may be other potential $\alpha_6\beta_1$ -HSPG-binding sites not yet identified, or that the H1 and H2 heparin binding sites may be sufficient to support cell adhesion through the $\alpha_6\beta_1$ -HSPG coreceptor complex.

EXAMPLE 11

SM Assay-Disrupted T1 binding site, inhibition of heparin binding sites

[0091] Because SM retains intact heparin binding sites, we tested whether cell adhesion to SM is dependent on HSPGs. We treated fibroblasts with either heparinase I (2 units/ml) or chondroitinase ABC (2 units/ml) (both from Sigma, St. Louis, MO) prior to adhesion to microtiter wells coated with WT CCN1 (2 $\mu\text{g/ml}$), SM (2 $\mu\text{g/ml}$) or VN (0.5 $\mu\text{g/ml}$). Control

cells were left untreated. Where indicated, soluble heparin was present at 1 $\mu\text{g/ml}$ in the culture medium:

[0092] As expected, either the presence of soluble heparin that saturates the CCN1 heparin binding sites, or treatment of cells with heparinase I to damage cell surface HSPGs was able to block cell adhesion to WT CCN1. *See* Chen, N., Chen, C. C., and Lau, L. F. (2000) *J. Biol. Chem.* **275**, 24953-24961. Likewise, cell adhesion to SM was similarly inhibited by soluble heparin and heparinase treatment, indicating that interaction of cell surface HSPGs with SM is critical for cell adhesion to this CCN1 mutant. Treatment of cells with chondroitinase ABC had no effect, confirming the specific involvement of HSPGs rather than chondroitin sulfate proteoglycans in CCN1 actions.

EXAMPLE 12

DM Assay – Uninterrupted T1 binding site, disrupted heparin binding site

[0093] To further assess the role of heparin binding in CCN1 functions, we examined the effects of eliminating heparin binding activity using DM mutants as described at Example 9. Mutations in the basic residues of H1 or H2, reduced heparin binding, and combining mutations in both H1 and H2 as shown in Fig. 9A obliterated heparin binding activity in CCN1 (Chen, N., Chen, C. C., and Lau, L. F. (2000) *J. Biol. Chem.* **275**, 24953-24961). Because DM has lost heparin binding activity but retains the T1 $\alpha_6\beta_1$ binding site, we postulated that DM would be compromised in $\alpha_6\beta_1$ -coreceptor-mediated activities, but might be able to support cell adhesion through $\alpha_6\beta_1$ at high coating concentrations analogous to cell adhesion to GST-T1 peptide fusion proteins (Fig. 9B).

[0094] Indeed, whereas cell adhesion to CCN1 reached a plateau at 1 $\mu\text{g/ml}$ (Fig. 10A), DM failed to support cell adhesion unless coated at much higher concentrations as an adhesive substrate, with maximal adhesion occurring at 50-100 $\mu\text{g/ml}$ (Fig. 11A).

EXAMPLE 13**TM Assay – Disrupted T1 binding site, disrupted heparin binding site**

[0095] As described in Example 9, we also created a mutant (TM) that combined the K293E mutation in the DM background, thereby disrupting the T1 binding site for $\alpha_6\beta_1$ as well as the H1 and H2 binding sites for heparin.

[0096] Strikingly, TM was unable to support cell adhesion at any concentration tested, remaining completely ineffective even when coated at 250 $\mu\text{g/ml}$. This observation indicates that DM may be able to support cell adhesion specifically through $\alpha_6\beta_1$, and this activity is eliminated by the K239E mutation in the T1 binding site for $\alpha_6\beta_1$.

EXAMPLE 14**Use of anti-integrins to evaluate cell adhesion of DM**

[0097] To verify the elimination of $\alpha_6\beta_1$ adhesion activity by the K239E T1 mutation, we examined whether DM-supported cell adhesion was mediated through $\alpha_6\beta_1$. We preincubated cells with 40 $\mu\text{g/ml}$ of function-blocking mAb against integrin $\alpha_v\beta_3$ (LM609), integrin α_6 (GoH3), or integrin β_1 subunit (P4C10, 1:50 ascites) at room temperature for 1 h prior to plating. We then assessed cell adhesion as described above.

[0098] Consistently, anti-integrin α_6 (GoH3) or β_1 (P4C10) mAbs blocked DM- or CCN1-supported fibroblast adhesion, whereas the anti- $\alpha_v\beta_3$ mAb LM609 had no effect (Fig. 11B). Together, these results show that fibroblast adhesion to CCN1 requires the $\alpha_6\beta_1$ -HSPG coreceptors interacting with three binding sites: T1, H1, and H2. Mutations in H1 and H2 (DM) still allow cell adhesion through $\alpha_6\beta_1$ at high coating concentrations, whereas disruption of all three sites (TM) completely abolishes cell adhesion through $\alpha_6\beta_1$ -HSPGs (Fig. 11A).

EXAMPLE 15**MAPK activation**

[0099] CCN1 has the unusual ability to induce sustained activation of p42/p44 MAPKs as an adhesion substrate (Chen, C.-C., Chen, N., and Lau, L. F. (2001) *J. Biol. Chem.* 276, 10443-10452). To test the effect of mutations in the T1, H1 and H2 binding sites for this activity, fibroblasts were adhered to WT CCN1, SM, DM, or laminin for various durations (Fig. 12A).

To evaluate MAPK activation, 1064SK fibroblasts were serum-starved, resuspended in serum-free medium at 6×10^5 cells/ml and plated on 35 mm dishes pre-coated with CCN1 (10 μ g/ml), SM (10 μ g/ml), DM (250 μ g/ml), or laminin (10 μ g/ml) for 1-5 hrs as indicated. Clarified cell lysates were electrophoresed on 10% SDS-PAGE and immunoblotted with polyclonal anti-MAPK antibodies, or antibodies specific for dually phosphorylated p42/p44 MAPKS (Progenia, Madison, WI).

[0100] As shown in Fig. 12A, cells adhered to laminin resulted in a rapid and transient activation of p42/p44 MAPKS typical of cell adhesion to ECM substrates, with activation reaching maximal level 1 hr after plating and declining to background level thereafter. In contrast, activation of MAPKS in cells adhered to WT CCN1 or SM was sustained to a large extent even 5 hrs after plating. This prolonged MAPK activation was lost in cells adhered to DM. Thus, mutations in the H1 and H2 sites had no effect on short-term activation of MAPKS, but specifically abrogated the ability of CCN1 to induced sustained MAPK activation.

EXAMPLE 16

Regulation of gene expression

[0101] CCN1 activates a genetic program in fibroblasts, leading to the upregulation of genes encoding proteins involved in angiogenesis and matrix metabolism, including the angiogenic inducer VEGF and matrix metalloproteinase MMP-1 (Chen, C.-C., Chen, N., and Lau, L. F. (2001) *J. Biol. Chem.* 276, 10443-10452). Because activated MAPKS are translocated into the nucleus where they can phosphorylate and activate transcription factors (Hazzalin, C. A. and Mahadevan, L. C. (2002) *Nat. Rev. Mol. Cell Biol.* 3, 30-40), the loss of sustained MAPK activation may alter the ability of CCN1 to regulate gene expression.

[0102] To test the loss of sustained MAPK activity on the ability of soluble CCN1 (WT) and CCN1 mutants (SM, DM, TM) to regulate gene expression, we treated serum-starved primary human skin fibroblasts with 10 μ g/ml of protein in serum-free condition for 24 hrs. Total cellular RNA was isolated, resolved on agarose-formaldehyde gel and blotted onto nylon membrane using standard protocols. We generated radioactive cDNA probes by random primer labeling incorporating 32 P-dCTP into human VEGF-A, MMP-1, and GAPDH cDNAs as described (Chen, C.-C., Chen, N., and Lau, L. F. (2001) *J. Biol. Chem.* 276, 10443-10452). We

then washed the blots at high stringency (0.1 x SSC; 0.1% SDS at 65°C) and analyzed them using Phosphorimager (Molecular Dynamics, Sunnyvale, CA). Expression of *Vegf* and MMP-1 was evaluated by RNA blotting (20 µg of total RNA in each lane) following electrophoresis, and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression was monitored as a control.

[0103] As shown in Fig. 12B, SM was able to upregulate *Vegf* and MMP-1 expression similar to WT CCN1, whereas DM and TM were completely defective in this activity. Thus, the heparin binding sites H1 and H2 are required for both prolonged activation of MAPKs and upregulation of *Vegf* and MMP-1 expression.

EXAMPLE 17

HUVEC adhesion and migration to CCN1 mutants through integrin $\alpha_v\beta_3$

[0104] We examined the adhesion of activated HUVECs to CCN1 mutants. HUVECs detached in 2.5 mM EDTA and resuspended in serum-free medium were adhered to wells pre-coated with 15 µg/ml of wild type CCN1 (WT) or SM, or 50 µg/ml of DM or TM. Where indicated, we treated the cells with EDTA (5 mM), GRGDSP peptide (RGDS, 0.2 mM), anti- $\alpha_v\beta_3$ mAb LM609 (40 µg/ml), anti- α_6 mAb GoH3 (40 µg/ml) for 60 min prior to plating. Cell adhesion was measured as described.

[0105] Both WT CCN1 and SM supported cell adhesion to a similar extent (Fig. 13A). HUVEC adhesion to both WT and SM were completely inhibited by EDTA and partially inhibited by GRGDSP peptide or the anti- $\alpha_v\beta_3$ mAb LM609, indicating the involvement of integrin $\alpha_v\beta_3$. Cell adhesion was also partially inhibited by the anti- α_6 mAb GoH3, consistent with a contribution from the $\alpha_6\beta_1$ -HSPG coreceptors (Leu, S.-J., Lam, S. C. T., and Lau, L. F. (2002) *J. Biol. Chem.* 277, 46248-46255). In contrast, HUVEC adhesion to DM and TM was completely inhibited by EDTA, GRGDSP peptide and LM609, but not at all by GoH3. These results indicate that DM and TM can support activated HUVEC adhesion through $\alpha_v\beta_3$, but have lost the ability to support $\alpha_6\beta_1$ -mediated cell adhesion.

EXAMPLE 18**Migration of HUVECs to CCN1 or mutants**

[0106] Using a modified Boyden chamber assay, we found that WT CCN1 and mutants in the $\alpha_6\beta_1$ -HSPG binding sites were able to stimulate HUVEC migration (Fig. 13B). Migration of HUVECs to CCN1 or mutants were evaluated using Transwell chambers. 15 $\mu\text{g/ml}$ of CCN1 WT or mutant SM, 50 $\mu\text{g/ml}$ of mutant DM or TM was immobilized on the lower surface of the Transwell membrane that separated the two chambers. HUVECs were treated with 100 nM PMA for 30 min to activate integrin receptors. Where indicated, prior to plating in the upper chamber, cells were preincubated with vehicle buffer (No Add), normal mouse IgG (100 $\mu\text{g/ml}$) (Sigma, St. Louis, MO), GoH3 (50 $\mu\text{g/ml}$) (Chemicon, Temecula, CA), LM609 (50 $\mu\text{g/ml}$) (Immunotech, Marseille, France) for another 30 min. Cells were allowed to migrate for 8 h, and those migrated to the lower chamber were counted in 10 random high power fields.

[0107] Cell migration to CCN1 and all mutants was completely inhibited by the anti- $\alpha_v\beta_3$ mAb LM609, whereas the anti- α_6 mAb GoH3 had no effect. Thus, CCN1 mutants with disrupted $\alpha_6\beta_1$ -HSPG binding sites are capable of stimulating $\alpha_v\beta_3$ -dependent HUVEC migration, similar to WT.

EXAMPLE 19**Enhancement of VEGF-induced DNA Synthesis through integrin $\alpha_v\beta_3$**

[0108] CCN1 enhances growth factor-induced DNA synthesis without being mitogenic on its own. See Kireeva, M. L., Mo, F.-E., Yang, G. P., and Lau, L. F. (1996) *Mol. Cell. Biol.* 16, 1326-1334. We preincubated HUVECs with vehicle buffer (No Add), LM609 (25 $\mu\text{g/ml}$), GoH3 (25 $\mu\text{g/ml}$), or normal mouse IgG (25 $\mu\text{g/ml}$) for 1h. We then treated the cells with VEGF (5 ng/ml) and/or CCN1 or mutant proteins (5 $\mu\text{g/ml}$ each) in the presence of [^3H]thymidine, incorporation of which was assessed 48 h thereafter. Data shown are mean \pm S.D. of three determinations and representative of three experiments.

[0109] As expected, VEGF (5 ng/ml) treatment induced DNA synthesis in HUVECs, whereas addition of CCN1 or mutants by themselves had no effect (Fig. 14). Treatment of cells with CCN1, SM, DM or TM in the presence of VEGF, however, enhanced VEGF-induced DNA synthesis by ~ 2 -fold. LM609 blocked this enhancement, thus reducing DNA synthesis to the

level induced by VEGF alone. In contrast, neither control IgG nor GoH3 had any effect. Thus, SM, DM and TM are all able to enhance growth factor-induced DNA synthesis in endothelial cells through integrin $\alpha_v\beta_3$, similar to WT CCN1.

EXAMPLE 20

CCN1 mutants promote HUVEC survival

[0110] We have previously established that CCN1 promotes endothelial cell survival under conditions of growth factor deprivation through integrin $\alpha_v\beta_3$. See Leu, S.-J., Lam, S. C. T., and Lau, L. F. (2002) *J. Biol. Chem.* 277, 46248-46255. To ascertain whether the CCN1 mutants can promote cell survival, we examined their effects in HUVECs plated on laminin using a TUNEL assay (Fig. 15). Serum-starved HUVECs were allowed to attach to coverslips pre-coated with 20 $\mu\text{g/ml}$ laminin (LN) for 4 hr. We then added serum, CCN1 or mutant proteins (5 $\mu\text{g/ml}$ each) for an additional 16 h. Cells were fixed and apoptosis was monitored by using a TUNEL assay. Where indicated, we preincubated polyclonal anti-CCN1 antibodies with test reagents for 30 min prior to addition into medium.

[0111] Very few apoptotic cells were detected when cells were maintained in 20% serum, whereas >70% of cells were apoptotic in serum-free medium. The addition of CCN1, SM, DM, or TM all reduced the number of apoptotic cells by ~40%. This effect was reversed by the presence of anti-CCN1 antibodies, indicating that promotion of cell survival is an activity of the CCN1 polypeptides. HUVECs treated under the same conditions were also monitored for DNA synthesis (data not shown). Consistent with results shown in Fig. 14, the rate of DNA synthesis was unaffected by the presence of CCN1 or mutants alone, indicating that the increase of non-apoptotic cells was not due to increased cell proliferation. These results show that the CCN1 mutants are still able to promote endothelial cell survival under conditions of growth factor deprivation, an activity mediated through $\alpha_v\beta_3$.

EXAMPLE 21

Tubule formation

[0112] CCN1 also induces tubule formation in activated endothelial cells through integrin $\alpha_v\beta_3$ when cultured in collagen gel. See Leu, S.-J., Lam, S. C. T., and Lau, L. F. (2002) *J. Biol. Chem.* 277, 46248-46255. To examine this activity in the CCN1 mutants, HUVECs were either treated

with vehicle buffer (no add) or stimulated with 5 nM PMA in serum-free medium before being plated on 24-well plates pre-coated with type I collagen gel (2 mg/ml) in the absence (-) or presence of CCN1 (WT) or TM (20 µg/ml each). A second layer of gel of identical formulation was overlaid on the attached cells, and tubule formation was assessed 16 h thereafter. Where indicated, LM609 (40 µg/ml) or GoH3 (40 µg/ml) was added to cell suspensions prior to plating. Results are representative of three experiments (x 200 magnification).

[0113] Unstimulated HUVECs showed no tubule formation in the absence of CCN1, and 20 µg/ml CCN1 did not induce tubule formation (Fig. 16). PMA-activated HUVECs, however, responded to WT CCN1 or TM by forming tubules. Addition of LM609 (40 µg/ml) to cell suspension prior to plating inhibited tubule formation, whereas addition of GoH3 (40 µg/ml) had no effect. The same results were obtained with SM and DM (Data not shown). These results show that CCN1 mutants are capable of inducing $\alpha_v\beta_3$ -dependent tubule formation in activated endothelial cells.